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RESEARCH ARTICLE

"Glutamate Excitotoxicity: An Insight into the Mechanism".

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Abstract

Blatant role of glutamate in physiology and neurologic disorders has made it challenging to know its chemistry and metabolic mode of action. As glutamate is ubiquitously present in the brain, it is involved in number of essential phenomenons associated with neural functioning. Among all the processes excitotoxicity is of great significance and consequence. Glutamate excitotoxicity refers to neuronal dysfunction and degeneration caused by over activation of glutamate receptors. This overview apostrophizes in brief glutamate from neural transmission to excitotoxicity, engrossing the structure and physiology of glutamate receptors and plausible cellular and molecular mechanisms underlying excitotoxic cell death. Considerable acquaintance with this intriguing phenomenon will enhance the understanding of the neuropathologic processes and help to develop a conceptual model for modulating excitotoxicity and emerging targets for excitotoxicity research.
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INTRODUCTION

Broadly Glutamic acid (Glu or E) is a proteinogenic amino acid, coded by GAA and GAG codon. It is a non essential amino-acid, chemically known as 2-Aminopentanedioic acid or 2-Aminoglutaric acid. Glutamic acid was discovered and identified for the first time by the German chemist Karl Heinrich in 1866 on treating wheat gluten with sulfuric acid. In 1907 Japanese researcher Kikunae Ikeda identified brown crystals of glutamic acid formed after the evaporation of a large volume of kombu broth. He patented the method of mass-production of crystalline salt of glutamic acid, monosodium glutamate (MSG). As characteristics of typical amino acids, glutamate consists of a central carbon atom bonded to a carboxyl group (COOH) and an amino group (NH3) and a distinctive side chain CH2CH2COO- (R group), which characterizes each amino acid, linked to the central carbon . At pH greater than pKa (pH>4.1), glutamate exists as negatively charged deprotonated carboxylate form due to its side chain carboxylic acid functional group (pKa 4.1). The carboxylate anions and salts of glutamic acid are known as glutamates. Glutamic acid occurs naturally in many foods. It was initially identified as a flavouring agent. Monosodium glutamate (MSG) is used as a food additive and flavour enhancer. Later on it was found that glutamate is a key compound in cellular metabolism (Lehninger, 2005) . It plays a pivotal role in transamination and deamination of amino acids. Transamination of α-ketoglutarate an intermediate in the citric acid cycle gives glutamate and other α-ketoacid which is often useful as fuel or as a substrate for further metabolic processes. Glutamate also undergoes deamination, and helps in the body's disposal of excess or waste nitrogen in form of ammonia which is then excreted predominantly as urea . Glutamate produced during transamination thus undergoes deamination, effectively allowing nitrogen from the amine groups of amino acids to be removed, via glutamate as an intermediate, and eventually excreted from the body in the form of urea.

Glutamate is one of the 20 amino acids known to be used in protein synthesis and metabolic functions like energy production and ammonia detoxification. However, it was hard to believe that a compound with so many functions, present everywhere in high concentrations could play an additional role as transmitter in nervous system. Although it was noted already 70 years ago that glutamate is abundant in the brain and plays a central role in brain metabolism, it took researchers

a long time to realize that glutamate is a neurotransmitter. Moreover, glutamate can serve as a source of energy for the brain cells in absence of glucose and serves as a link between the redox states of the pyridine nucleotides (NAD+ and NADP+). On an average per kg of brain tissue contains 5 - 15 mM glutamate (Colin, 2008). Glutamate has the greatest ascendancy as excitatory neurotransmitter in vertebrate central nervous system. It is also most abundant free amino acid in brain. It is involved in most aspects of normal brain function including cognition, memory and learning (Okubo et al., 2010). Glutamate also serves as the precursor for the synthesis of closely related glutamine via glutamine synthetase, and to γ-aminobutyric acid (GABA) via glutamate decarboxylase. Glutamate not only mediates information, but also regulates brain development and cellular survival, differentiation and elimination as well as synapses i.e. nerve contacts (McDonald and Johnston, 1990). Glutamate in cerebrum is derived solely from local synthesis from L-glutamine and α-ketoglutarate, which is a product of the Krebs cycle. However, considerable fraction is also derived from recycling of brain proteins. Role of glutamate being in both metabolism and in neurotransmission suggests that a higher level of regulation is required of glutamate concentrations in central nervous system (CNS). Glutamate concentrations in blood undulate due to changes in diet, metabolism and protein turnover. If these fluctuations are transferred directly to the brain they would have disorganizing effects on neuronal transmission. One of the important mechanisms for regulating glutamate concentrations in brain is blood brain barrier (BBB). The BBB forms an impediment, separating brain extracellular fluid from circulating blood. It consists of a single layer of endothelial cells and multiple bands of tight junctions which seal of the paracellular diffusion spaces restricting the diffusion of various solutes into the cerebrospinal fluid (van Meer et al., 1986). Most substrates are transported across BBB either by dissolving through it or are transported across by selective carriers or membrane transporters present on BBB (M., 1998). These transporters regulate the flux of solutes such as glutamate between blood and brain interstitial fluid. High levels of more than 20 such transport systems are present on BBB cells for such regulation. Glutamate is synthesized promptly in brain, thus it shows much lower rates of influx into brain at the BBB. About nine amino acid transport systems are present at the BBB capillary endothelium, among them transporter namely System X2, mediates sodium-independent (Na+-independent), high affinity uptake of glutamate. Glutamine on the other hand is taken up into the brain by a separate Na+-dependent mechanism known as System N and/or ASCT2 transporters (Chaudhry et al., 1999). Another possible route for glutamate transport into brain is high affinity glutamate transport system present at the choroid plexus epithelium (Preston and Segal, 1992). Extracellular concentrations of brain glutamate may be additionally regulated by an active efflux pump which may exist at the blood-brain barrier. Although the BBB regulates glutamate in most of the brain from changes in circulating plasma glutamate, however few brain areas lack BBB leading to rapid glutamate uptake from the circulation(Hawkins et al., 1995) . These areas are called as circumventricular organs (CVO's) and include the median eminence, area postrema, subfornical organ, subcommissural organ, pineal gland, and neurohypophysis and organum vasculosum of the lamina terminalis (Gross et al., 1987). The CVOs act as direct link between the central nervous system and peripheral blood flow allowing uptake of small solutes at rates 10-1000 times higher than those of normal brain (Shaver et al., 1992). Intercellular diffusion from CVO's into adjacent brain areas results in vulnerable to acute fluctuations in concentration of various solutes (Price et al., 1984).

Excitatory Amino Acid Receptors

Brain consists mainly of two types of cells, neurons and glial cells. Neurons show Ca2+ dependent synaptic vesicular release of glutamate. By contrast glial cells release glutamate in different ways. These include vesicular release, reverse operation of the Na+-dependent glutamate transporters, swelling activated anion channels or through hemichannels (Montana et al., 2006). Glutamate is synthesized within the neurons in a highly organized and coordinated manner. It is processed among various cytoplasmic organelles and then transported outside in form of vesicles (Siegel et al., 1994). Glutamate is first synthesized by the endoplasmic reticulum and then transported to the Golgi apparatus. From Golgi apparatus glutamate emerges from the opposite surface in form of bilipid membrane vesicles. These vesicles are then transported down the axon via a complex system of microtubules. Vesicular motion is mediated by antegrade motion of molecules called motor kinesin, whereas cytoplasmic dynein generates retrograde motion (Mark et al., 2001). The energy required for the motion is provided by mitochondria which escort these transport molecules. Upon reaching the axonal tip these vesicles coalesce with the presynaptic membrane by the process called exocytosis to release the glutamate into the synaptic space between neurons. Glutamate stored in pre-synaptic vesicles is released via a Ca2+-dependent mechanism, to interact, bind and activate postsynaptic glutamate receptors. The vesicular membrane is then recycled and transported back up the neuronal axon via the microtubular network. Once released, glutamate can bind to a number of receptors that mediate different biological responses. After its interaction with postsynaptic receptors it is quickly removed from the synaptic cleft by neuronal presynaptic and glial high affinity reuptake systems (Kanai et al., 1993).As a consequence a 5000-10,000-fold gradient of glutamate is maintained from the intracellular to the extracellular space (Ferrarese et al., 1993). The most important event after the release of glutamate in the postsynaptic membrane is its interaction with glutamate receptors. There are two main types of glutamate receptors ionotropic and metabotropic. Ionotropic receptors are directly coupled to membrane ion channels. The metabotropic receptors are coupled to intermediary compounds, such as G protein, and modulate intracellular second messengers, such as inositol-1, 4, 5-trisphosphate (IP3), Ca2+, and cyclic nucleotides(Pin and Duvoisin, 1995). The ionotropic receptor can be further subdivided into three subtypes. N-methyl-D-

aspartate (NMDA), amino-3-hydroxy-5-methyl-4-isoxazolpropionate (AMPA) and kainate receptors .These subtypes are named for their selective chemical agonists, which resemble glutamate but do not naturally occur in the brain. All three ionotropic receptor families are mediated by the opening of cation channels permeable to Na+ and in a subtype-specific way to Ca2+, thereby depolarizing or "exciting" the neuron. The ion channels of the AMPA and kainate receptors allow monovalent ions to pass, whereas NMDA receptors allow the inflow of both Na+ and Ca2+ ions. AMPA and kainate receptors play the primary role in mediating fast excitatory postsynaptic potentials responsible for excitatory neurotransmission (Dawson et al., 1994).

Ionotropic Receptors

(I) N-Methyl-D-Aspartate Receptors (NMDARs): The classical properties attributed to NMDARs include glutamate activation, magnesium block, zinc inactivation, glycine activation, interactions with polyamines and pH sensitivity. The NMDARs have been implicated in various processes from learning and memory to neurodegeneration. The NMDAR channel is made up of a combination of three different subunits known as NR1, NR2 and NR3. The traditional NMDAR is heterotetramer composed of two NR1 subunits and two NR2 subunits. The NR1 subunit is made up of ~938 amino acids and has eight splice variants. NR2 exists in four subtypes (NR2A–D).The major role of NR2 subunits in NMDAR function is of regulatory and refining (Luo et al., 2011). Within the NMDAR complex, NR2 subunits modulate the characteristics of the NR1 ion channel pore. NR2A is widely distributed in the brain, NR2B primarily in the forebrain, NR2C predominantly in the cerebellum and NR2D is localised to the thalamus. NR2B subunits create specialised postsynaptic microenvironments by binding and linking postsynaptic proteins (Sattler et al., 1999). The NR3subunit is expressed in two isoforms: NR3A, which is expressed throughout the CNS, and NR3B, which is expressed primarily in motor neurons. When activated, the NMDAR allows the influx of cations followed by the activation of intracellular pathways leading to physiological processes such as learning and memory as well as pathological processes. The NMDAR works through a complex gating mechanism, which involves binding of various ligands and cellular depolarisation. At resting membrane potential channel is blocked by Mg2+. Depolarisation of the NMDAR-expressing neuron is necessary to electrostatically remove Mg2+.Upon depolarization Mg2+ block is removed, permitting glutamate to open the NMDA channel. The channel is permeable not only to Na+ but also to Ca2+, an important intracellular signaling ion that activates nitric oxide synthetase, among other enzymes. The NMDA receptor is unique among all known neurotransmitter receptors, as two agonists must be present simultaneously for activation to take place: glutamate and glycine (Klein and Castellino, 2001). Under normal conditions glycine modulatory site is not fully occupied (Danysz and Parsons, 1998). NMDARs are recruited only during periods of substantial neuronal depolarization; it appears to serve the purpose of detecting concomitance. In this way, the NMDA receptor plays a critical role in a major form of use-dependent synaptic plasticity known as long-term potentiation linked to memory formation (Bliss and Collingridge, 1993). Glutamate or alternatively zinc inhibits the NMDAR-mediated currents elicited by glutamate (Mayer and Vyklicky, 1989) .Preliminary evidence suggests that NR1/NR3A and NR1/NR3B complexes are not activated by NMDA or glutamate, but rather elicit an excitatory Ca2+ impermeant response via glycine. In addition to agonist binding sites NMDA receptor has a number of modulatory sites that affect its activity. Binding site for the dissociative anesthetics which serves as noncompetitive antagonists such as phencyclidine (PCP, "angel dust") and ketamine are also present within the channel. Another binding site insensitive to strychnine, for co-agonist glycine must be occupied for opening of glutamate ion channel (Huettner, 1991). This site on the NMDA receptor is distinct from the strychnine-sensitive site associated with the inhibitory glycine receptor in the brainstem and spinal cord. The polyamine and NMDAR interaction is less clear and many of these effects are dependent on the subunit constituents of the NMDAR channel (Williams, 1997). As such, the physiological significance of these interactions is still under investigation. Spermine, a polyamine, at low concentrations potentiates NMDAR currents, but at higher concentrations it reduces current in a voltage-dependent manner (Rock and Macdonald, 1992). Alterations in physiologically pH due to various neurological insults can also modulate NMDAR function.

(II)Amino-3-Hydroxy-5-Methyl-4-Isoxazol-Propionic Acid Receptors (AMPARs): AMPA receptors are made up of a combination of four subunits (GluR1–GluR4) encoded by four different genes. The molecular diversity of AMPA receptors is enhanced by splice variants, in which mRNA is constructed from different exons, resulting in a number of physiologically distinct receptor channels from the same gene and from posttranscriptional processing of mRNA .AMPARs require only glutamate application for activation and are localised mostly in the postsynaptic membrane. The specificity of cation influx of AMPARs is variable and is governed primarily by subunit composition. GluR1, GluR3 and GluR4 all display strong inwardly rectifying current–voltage and Ca2+ permeability, whereas the GluR2 subunit removes Ca2+ permeability (Burnashev et al., 1995; Vignes and Collingridge, 1997). Physiologically, AMPARs are thought to regulate the fast excitation required to remove the Mg2+ block of nearby NMDARs.

(III)Kainate Receptors: Three genes encode the family of kainate receptors GluR5–7 also known as GluK5–7, whereas two additional genes encoding polypeptides KA1 and KA2 also known as GluK1–2 alters the pharmacologic features of the kainate receptors. The properties of kainate channels are similar to AMPARs in that they allow ion flux directly

following glutamate application, though they are mostly impermeant to Ca2+ ions. Kainate receptors are localised in both the presynapse and post-synapse . Some studies have shown that the application of kainate can stimulate glutamate release, whereas others have shown that kainate application inhibits presynaptic glutamate release (Schmitz et al., 2001). Postsynaptically, kainate channels serve a similar purpose as AMPARs in alleviating Mg2+ block in NMDARs.

Metabotropic Glutamate Receptors

Metabotropic glutamate receptors (mGluRs) are single peptide seven-transmembrane spanning proteins linked to intracellular G-proteins. Metabotropic receptors change intracellular processes via signal transduction mechanisms and in this way intervene in a wide variety of different cellular functions. It was originally believed that all metabotropic glutamate receptors do not have ion channels, but mediate their function via G-proteins , which then in turn trigger further second-messenger systems, however recent evidences suggests that G-protein-independent signaling can occur (Heuss et al., 1999). Eight different types of mGluRs are known (mGluR1–8). All mGluR subtypes are inhibited by coupling of adenylate cyclase via their G-protein, which causes the synthesis of the intracellular second messenger cAMP to be suppressed. Metabotropic glutamate receptors (mGluR) are classified into 3 groups on the basis of sequence homology, intracellular effects, coupled second messenger systems and pharmacological properties. The group I family of mGluRs is associated with the post-synapse and appears to potentiate NMDAR-mediated Ca2+ influx. The remaining metabotropic glutamate receptor heterodimers are linked to the inhibition of cAMP formation. These receptors are primarily found in the pre-synapse and reduce Ca2+ influx via NMDARs (Bruno et al., 1995).

(I)Group I Metabotropic Glutamate Receptors: These include mGluR1s and mGluR5s coupled to Gq/G11, pharmacologically activated by 3, 5-dihydroxyphenylglycine (DHPG). The Activation of group I mGluRs is linked via G-proteins to the activation of phospholipase C (PLC), which hydrolyses phosphoinositols (PI) into the intracellularly effective second messengers inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 releases Ca2+ from intracellular stores whereas DAG activates all isotypes of protein kinase C (PKC). However at hippocampal synapses this group of mGluRs modulates excitatory postsynaptic potentials via tyrosine kinases in a G-protein-independent fashion. Both receptor subtypes are localized in the extrasynaptic portion of dendritic spines, where they are anchored to NMDA receptors via a chain of scaffolding proteins (Tu et al., 1999). Homer proteins link the C-terminus domain of mGlu1a and mGlu5 receptors to a number of membrane and intracellular proteins, including signaling proteins (e.g., phospholipase-Cb3, and the PtdIns-3-kinase enhancer, PIKE-L) and ion channels (e.g., NMDA receptors, InsP3 receptors, and TrpC6 channels) (Brakeman et al., 1997). PIKE-L links mGlu1 and mGlu5 receptors to the activation of the PtdIns-3-kinase (PI3K) pathway, involved in neuronal survival through the inhibition of glycogen synthase kinase-3b (GSK3b) and other mechanisms (Brazil and Hemmings, 2001). mGlu5 receptors in particular facilitate the activation of NMDA receptors by relieving the Mg2+ blockade of the NMDA-gated ion channel or through other mechanisms (Mannaioni et al., 2001).Sustained increases in intracellular Ca2+ levels, as occurs in response to a toxic activation of NMDARs, cause a calpain-mediated cleavage of the C-terminal portion of the mGlu1a receptor.mGlu1a receptors can both stimulate intracellular Ca2+ release and activate the PI3K pathway only if the C-terminus domain is intact. Thus this cleavage prevents the receptor from activating the PI3K pathway while leaving its ability to stimulate PtdIns-4, 5-P2. In contrast, glutamate-bound mGlu1 receptors involves a novel G-protein independent pathway mediated by b-arrestin1-dependent, persistent activation of the MAPK pathway (Emery et al., 2010; Pshenichkin et al., 2008). Competitive mGlu1 receptor antagonists block the stimulation of PtdIns-4, 5-P2 hydrolysis but not the prosurvival signal triggered by glutamate (Pshenichkin et al., 2011).

(II)Group II Metabotropic Glutamate Receptors: These include mGluR2s and mGluR3s coupled to Gi/Go proteins, potentially activated by a series of conformationally constrained glutamate analogues such as compounds (1S,2S,5R,6S)-(+)-2-aminobicyclo- [3.1.0]hexane-2,6-dicarboxylic acid (LY354740) and (_)-2-oxa-4-aminocyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268)(Schoepp et al., 1999). Both mGlu2 and mGlu3 receptors are preferentially localized in the preterminal region of axon terminals, where they negatively regulate neurotransmitter release. mGlu3 receptors are also found in glial cells , where their activation enhances the synthesis and secretion of neurotrophic factors. The excitation of mGluR from this group leads to an inhibition of adenylate cyclase, resulting in a reduced production of cyclic adenosine monophosphate and thus a reduced glutamate release, resulting in downstream inhibition of voltage-dependent Ca2+ channels, activation of K+ channels, and activation of the mitogen-activated protein kinase (MAPK) and the PI3K pathways(Chavis et al., 1994). These receptors are found at both the presynapse and the post-synapse. Since presynaptic Ca2+ is integral to neurotransmission, group II mGluRs modulate neurotransmission via their action on voltage gated Ca2+ channels.mGlu2 and mGlu3 receptors reduce neuronal excitability by activating different types of K+ channels and negatively regulate glutamate release (Nicoletti et al., 2011). Glial mGlu3 receptors regulate the production of neurotrophic factors other than TGF-b1, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial derived neurotrophic factor (GDNF) .

(III)Group III Metabotropic Glutamate Receptors: These mGluRs include mGluR4s, mGluR6s-mGluR8s coupled to Gi/Go proteins and activated by L-2-amino-4-phosphonobutanoate (L-AP4) and L-serine-O-phosphate (L-SOP). These mGluRs are also found in both the presynaptic and postsynaptic terminals, and similar to group II receptors, these mGluRs modulate neurotransmission by functioning as autoreceptors and are also associated with a decrease in adenylyl cyclase signaling, resulting in downstream inhibition of voltage dependent Ca2+ channels and inhibiting glutamate release (Niswender and Conn, 2010).

Excitatory Amino Acid Transporters (EAAT)

Following binding to synaptic glutamate receptors, glutamate is either transported back into presynaptic neuronal terminals or taken up into nearby glial cells. The principal mechanism for the maintenance of low extracellular glutamate is through Na+-dependent glutamate transporters that are anchored within the cell membranes of astrocytes and neurons named excitatory amino acid transporters (EAAT1 or EAAT2), also called Glt-1 and Glast. These transporters are abundant on astrocytic membranes and account for approximately 1% of total brain protein (Danbolt, 2001). EAATs are driven by the electrochemical gradients across the plasma membrane and require Na+ for glutamate binding and K+ as a driving force to take up extracellular glutamate against several thousand-fold concentration gradients, leading to an extracellular concentration below 1M (Gegelashvili and Schousboe, 1997). These transporters so far have five subtypes EAAT-1 - EAAT-5 (Fairman et al., 1995). EAAT-1 and EAAT-2 are primarily present in astrocytes. EAAT2 is a hexamer transmembrane protein predominantly expressed in astrocytes and is responsible for the majority of glutamate uptake in the human brain. EAAT-3 is a neuronal transporter with a somatodendritic location (Rothstein et al., 1994). These three EAAT‘s were primarily cloned in murine systems. EAAT-4 is expressed in the cerebellum and EAAT-5 in the retina. EAAT-4 and EAAT-5 first described in humans are localized in close proximity of glutamatergic synapses. In fact, throughout the central nervous system most glutamatergic synapses are tightly ensheeted by astrocytic processes placing their glutamate transporters near synaptic release sites. It is believed that this arrangement ensures the rapid and effective containment of glutamate spillage out of the synaptic cleft. Direct measurements indicate that astrocytic processes in perisynaptic regions experience glutamate concentrations as high as ~180 μM, yet glutamate typically does not exceed 1-3 μM elsewhere in the brain (Dzubay and Jahr, 1999).

Upon uptake into astrocytes, glutamate is converted rapidly into glutamine by glutamine synthase (GS), an astrocyte-expressed enzyme. Then it is secreted by astrocytes through eight transmembrane domain transporter known as N or ASCT2 (Attwell et al., 1993; Martinez-Hernandez et al., 1977). These transporters are pH independent, but depend on exchange of counter substrate such as alanine, serine or cysteine for efflux of glutamine. Its pattern of expression suggests that ASCT2 intervenes in situations where astrocytes proliferate as during glioma. SAT1/GlnT, a pH-sensitive transporter with 11 transmembrane domains, ensures this function (Varoqui et al., 2000). SN1 a transmembrane protein with 11 transmembrane domains mediates system N transport. This coupling permits glutamate passage in the extracellular compartment in form of glutamine, thus avoiding toxicity (Broer and Brookes, 2001). Glutamine thus released is taken up by nearby neurons through the system known as System “A”. Glutamine transported to neurons is hydrolyzed into glutamate and ammonia via the mitochondrial phosphate-dependent glutaminase and stored for secretion via the vesicular glutamate transporters (VGLUT) (Daikhin and Yudkoff, 2000).

Cystine-Glutamate Exchanger (System Xc)

System Xc is electroneutral amino acid carrier, Na+ independent and transports cystine into the cell in exchange for glutamate (Ye et al., 1999). It requires both extracellular cystine and intracellular glutamate to function. It is a dimeric protein composed of two units a catalytic (XCT) and a regulator subunit (4F2hc) CD98, required for membrane association of the transporter (Sato et al., 1999). The catalytic subunit has 12 transmembrane domains and belongs to HET class of amino acid transporters. XCT expression is upregulated in gliomas and in response to oxidative stress. Glial cells normally uptake glutamate through high capacity EAAT2 transporters which have a high affinity for extracellular glutamate (Ye and Sontheimer, 1999).As a consequence system Xc does not account for a significant glutamate release, as glutamate released through the antiporter in exchange for cystine uptake is quickly removed through reuptake by EAATs. The main cellular function of system Xc is the uptake of cystine for the generation of the cellular antioxidant glutathione (Chung et al., 2005). The cystine is reduced to cysteine which along with glutamate and glycine forms the tripeptide Glu-cysteine-glycine i.e. glutathione (GSH). GSH contains highly reactive SH groups that can readily bind to oxidative and nitrosylative radicals thus guarding the cells redox status (Iida et al., 1997). Metabolically active cells, and particularly cancers cells, often synthesize increased amounts of GSH presumably affording them a better protection towards endogenously produced radicals (Dun et al., 2006).

Role of Calcium (Ca2+)

In addition to glutamate transporters Ca2+ also plays a very crucial role in glutamate transport. Ca2+ transport is controlled by two main transporters, an antiporter and plasma membrane Ca2+ pump (PMCA). The antiporter helps in Ca2+ efflux from neurons by a Na+–Ca2+ exchange mechanism. It has a low affinity but high transport capacity for Ca2+ and is driven

by Na+ gradient across the membrane. A high Ca2+ concentration is maintained in the mitochondria by influx of free Ca2+ from the cytosol to the mitochondria. Ca2+ is sequestered in endoplasmic reticulum by an antiporter and an ATP-dependent active pump. The PMCA, on the other hand, has a high affinity but low transport capacity for Ca2+. This active pump transports one Ca2+ for each ATP hydrolyzed. PMCA can bind Ca2+/calmodulin; this binding enhances its affinity for Ca2+, which results in a 20- to 30-fold increase in the affinity of the substrate Ca2+ site. Intracellular Ca2+ levels are found to increase by direct opening of ion channels due to glutamate receptor overstimulation and secondarily affecting Ca2+ homeostatic mechanisms. Glutamate receptor initiated opening of the Na+ /Ca2+ channels allows the influx of Ca2+ and causes membrane depolarization which activates the voltage-dependent Ca2+ channels, which further increase the intracellular Ca2+ levels. The decreased Na+ gradient across the cell membrane caused by the glutamate receptor–coupled channels reduces the ability of the Na+ gradient–dependent antiporter to eliminate intracellular Ca2+.

Excitotoxicity-Cellular Mechanisms

Under certain conditions changes in glutamate homeostasis leads to its excessive release which causes overexcitation of cells causing neuronal dysfunction and degeneration, termed as excitotoxicity (Olney, 1969).As glutamate is a major excitatory neurotransmitter in the central nervous system (CNS), the implications of glutamate excitotoxicity are many and far-reaching. The term "glutamate excitotoxicity" was for the first time coined by Olney after observing that intracranial brain lesions developed in mouse models in response to subcutaneous injections of glutamate. The toxic effects of glutamate in mice model were also observed on inner layers of retina. Chronic over-excitation of neurons elicited by glutamate has linked glutamate excitotoxicity to neurodegenerative processes (Jorgensen and Diemer, 1982). Excitotoxicity is thought to be a major mechanism contributing to neurodegeneration in central nervous system disorders (Rothman, 1984). Excitotoxicity is thought to play an important role in the neural damage that occurs in diseases such as trauma, stroke, epilepsy and hypoglycemia. Glutamate excitotoxicity has been the implicated mechanism of neuronal injury in mesial temporal sclerosis with consistent, strong supportive experimental data and mitochondrial disorders, such as MELAS (mitochondrial myopathy, encephalopathy, lactacidosis, and stroke)(Hamberger et al., 1991). These and many more neurologic disorders are now assumed to share a common damaging metabolic pathway called excitotoxicity. Various glutamate receptors play a key role in mediating excitotoxic damage. The mechanisms underlying glutamate excitotoxicity are complex. However, especially in the acute pathologies, glutamate excitotoxicity is not thought to be the result of a genetic mutation or structural deficit in the channel. Abnormal release of glutamate from its storage sites in neuronal vesicles is at least one factor. Glutamate once released stimulates additional glutamate release by feedback loop mechanism. In case of energy failure glutamate reuptake is debilitated which affects glutamate transport out of the synaptic space and also causes the transporters to run backward, becoming a source of extracellular glutamate rather than a sink for it. In general, synaptic overactivity leads to the excessive release of glutamate.

One of the important attributes of glutamate excitotoxicity is Ca2+ influx. Neuronal culture studies have shown that Ca2+ rich extracellular environment increases glutamate excitotoxicity(Choi, 1987). During glutamate excitotoxicity intracellular Ca2+ is sequestered into mitochondria. Sequestration takes place by low affinity, high capacity Na+/Ca2+ exchanger buffering Ca2+ into mitochondrial stores. The glutamate-induced elevated Ca2+ levels triggers a sequence of different signals leading to neurotoxicity. Higher Ca2+ level overactivates a number of enzymes such as protein kinase C, Ca2+/calmodulin-dependent protein kinase II, phospholipases, proteases, phosphatases, nitric oxide synthase, endonucleases etc. Activation of such enzymes contributes to the excitotoxic cascade by increasing glutamate release through different signaling cascades. These enzymes and the generated feedback loops consequently lead to metabolic acidosis and free radical generation and finally to neuronal self digestion by protein breakdown, free radical formation, and lipid peroxidation. NMDARs were implicated to be primarily responsible for Ca2+ entry. However, it is thought that cascade which leads to Ca2+ influx is more important than Ca2+ load in the NMDAR-mediated neurodegenerative process. Ca2+ influxes via NMDARs are found to be more fatal than higher Ca2+ influxes via other Ca2+ permeant channels. Although much interest has focused on the role of Ca2+ in glutamate toxicity, it is found to occur by a mechanism that does not rely on Ca2+ influx. In this instance cell death correlated with swelling and blebbing, is dependent on ATP depletion and is independent of Ca2+. On the other hand, Ca2+ may play an important role in mitochondrial dysfunction as the mitochondria have the capacity to sequester Ca2+.As this occurs, however, there is a decrease in membrane potential that is proportional to the amount of Ca2+ taken up by mitochondria (Nicotera et al., 1990). The accumulation of large amounts of Ca2+ within mitochondria could have dire consequences, as their membrane potential could collapse, resulting in cessation of energy production. Another mechanism for increasing Ca2+ ions is via Na+–Ca2+ exchanger (NCX), a transmembrane protein that exchanges one Ca2+ ion per three Na+ ions. The direction of ion flux is dependent on a number of factors including pH, Na+ concentrations, Ca2+ concentrations and ATP levels. Under physiological conditions, the major driving force of these exchangers is the Na+ gradient created by the Na+/K+ ATPase. However, the direction of ion flux is reversible, and high intracellular Na+ levels can cause Ca2+ influx with Na+ extrusion increasing intracellular Ca2+ levels. In excitotoxicity, NMDAR-mediated Na+ influx is sufficient to reverse NCX function, causing Ca2+ accumulation in neurons. However, this phenomenon occurs in the presence of Na+/K+ ATPase dysfunction during severe insults (Czyz et al., 2002). Free radicals are found to be involved in glutamate toxicity. Various cell culture studies on excitotoxicity

demonstrated free radical production. The role of free radicals in excitotoxicity is an area of continuing interest. The mechanism of free radical production was initially coupled to Ca2+. Role of free radicals in glutamate excitotoxicity can be brought about from various studies. Groups have shown neuroprotection against glutamate excitotoxicity in antioxidant rich media cultures (Dykens et al., 1987). Similarly cultured cortical neurons overexpressing superoxide dismutase showed resistance to glutamate and ischemia-induced neurotoxicity (Gonzalez-Zulueta et al., 1998) . Recent in vitro studies showed that glutathione depletion exacerbates excitotoxicity in vitro while tocopherol, ascorbic acid and ubiquinone, which are free radical scavengers, exert neuroprotective effects (Puttfarcken et al., 1992). Role of zinc in neurodegeneration seems strong but elusive. One of the most important mechanisms of postsynaptic zinc accumulation including presynaptic zinc translocation, extracellular zinc influx, mobilization of zinc from the protein bound pool (especially from metallothioneins) via oxidative mechanisms and release from mitochondrial pools (Sensi et al., 2003).Zinc entry into neurons has been linked to voltage-gated Ca2+ channels, Na+ exchangers, NMDARs and AMPA/kainate receptors, but most recently, TRMP7 channels have been implicated as a novel route of zinc entry (Inoue et al., 2010). Zinc accumulation downstream of NO or NMDA application, requires reactive nitrogen species such as peroxynitrite to release intracellular zinc stores (Bossy-Wetzel et al., 2004). Under physiological conditions, zinc inhibits neuronal nitric oxide synthase, but can increase nNOS activity under pathological conditions possibly potentiating neuronal damage (Kim and Koh, 2002). More recent studies have shown that zinc accumulation following glutamate application is almost completely dependent on Ca2+ entry and subsequent ROS generation(Dineley et al., 2008).Direct evidence for zinc neurotoxicity was provided by extracellular exposure of cortical cultures to zinc which leads to concentration- and time-dependent modes of apoptotic or necrotic neuronal death (Choi et al., 1988; Yokoyama et al., 1986). Based on the histological changes observed in vivo, it was initially thought that the majority of the postsynaptic neurotoxic zinc was derived from the presynaptic terminals (Koh et al., 1996). However, later studies demonstrated zinc accumulation in the postsynaptic soma following insults even without presynaptic zinc stores (Lee et al., 2000). Currently, the evidence supports high intracellular levels of zinc have many detrimental effects.In mouse cortical neurons it inhibits GAPDH resulting in glycolytic dysfunction and energy failure (Sheline et al., 2000). It either inhibits TCA cycle directly or interferes in electron transport chain in isolated mitochondria (Brown et al., 2000). Mitochondrial dysfunction due to high levels of zinc leads to increase in reactive oxygen species. Many intracellular signaling cascades are disturbed due to high levels of zinc such as p38 phosphorylation, ERK 1/2 activation which leads to increased potassium influx and cell death (Du et al., 2002). Another potential mechanism of glutamate neurotoxicity is the generation of the second-messenger nitric oxide (NO). NO has generated much interest as the first of a novel class of neurotransmitters that acts as a second messenger after activation of NMDA receptors. NO was recently shown to mediate glutamate-induced neuronal toxicity in cultures of both cortical and striatial neurons (Dawson et al., 1991). A similar result was obtained in hippocampal slices, but was disputed in a study of hypoxia- and glutamate-induced cell damage in neuronal cultures (Izumi et al., 1992).

NO interacts with GAPDH, this interaction is found to be highly neurotoxic (Hara et al., 2005). Role of NO in glutamate-mediated neurodegeneration is backed by experiments in which nitric oxide synthase (NOS) inhibitors prevented cell death in in vitro cultures. Another connecting link between excitotoxicity and NO is formed by interaction of neuronal nitric oxide synthase (nNOS) with NMDARs. NMDARs are spatially linked to nNOS which can produce toxic levels of NO (Stamler et al., 2001). Overstimulation of NMDA receptors produces high levels of NO and superoxide ions. These free radicals react and form peroxynitrite.Peroxynitrite being extremely toxic and highly reactive results in neuronal death. NO can damage DNA; inhibit mitochondrial respiration, which in turn leads to formation of more free radicals causing membrane depolarization. NMDARs are bound to nNOS via a postsynaptic density protein of molecular weight 95 kDa (PSD-95) providing a structural link between nNOS and NMDARs. PSD-95 binds to the C-terminus of the NR2B subunit via a PDZ1 domain and the N-terminus of nNOS via a PDZ2 domain. A microenvironment is formed in the post-synapse whereby Ca2+ entering the neuron preferentially activate nNOS via calmodulin. NO has a number of intracellular targets such as the free radical superoxide to form peroxynitrite. Superoxide, is produced physiologically during normal cellular respiration, it is then catalyzed by superoxide dismutase into oxygen and hydrogen peroxide. In glutamate excitotoxicity, this buffering system is overwhelmed and superoxide spills into the cytoplasm. Peroxynitrite is also a powerful oxidative molecule which is a potent oxidant that can cause protein nitration, protein oxidation, protein dysfunction, lipid peroxidation and direct DNA damage leading to cell death (Radi et al., 1991). Specific interactions of peroxynitrite with proteins include protein oxidation nitrosylation (Alvarez et al., 1999). Finally, peroxynitrite can induce lipid peroxidation inhibit the mitochondrial electron transport chain at complex I and complex II, can inhibit the normal function of cytochrome c in the electron transport chain as well as manganese and iron superoxide dismutase in scavenging superoxide via protein nitration (Nakagawa et al., 2001). This interaction can potentiate caspase-mediated cell death and an eventual apoptotic cell death. Peroxynitrite-mediated DNA damage can cause overactivation of poly (ADP)-ribose polymerase (PARP-1), a nuclear repairing enzyme requiring NAD, leading to energy failure and necrotic cell death (Zingarelli et al., 1996). Peroxynitrite can also interact with proteases in other cell death pathways (Lau et al., 2006). A higher concentration of NO also leads to nitrosylation of GAPDH, a ubiquitous glycolytic enzyme. As a result GAPDH binds Siah1, an ubiquitin ligase. This heterodimer translocates to the nucleus with help of nuclear translocation domain present in Siah 1.In nucleus the complex enhances p300/CBP-associated acetylation of nuclear proteins leading to apoptosis. GOSPEL a cytosolic

protein has recently been shown to competitively inhibit Siah1 binding to GAPDH consequently leading to neuroprotection. NO also mediates GAPDH-GOSPEL binding. It leads to functional loss of GAPDH leading to necrotic cell death via energy failure.

Caspases, cysteine proteases are shown to play a role in delayed excitotoxic injury in cerebrocortical and cerebellar cultures (Tenneti and Lipton, 2000). Caspase inhibitors are found to provide neuroprotection from NMDA-mediated neurodegeneration in cultures. These studies also suggested that caspase activation occurs downstream of Ca2+ influx and mitochondrial dysfunction.

Calpains, cytoplasmic Ca2+ sensitive cysteine proteases have also been implicated in the pathogenesis of excitotoxicity. Cell cultures treated with calpain inhibitors provided neuroprotection from NMDA insults. However the role of calpains remains less clear .Calpains such as mu-calpain is necessary for the cleavage and release of apoptosis inducing factor (AIF) from mitochondria in a cell-free system (Polster et al., 2005). Cultures subjected to oxygen–glucose deprivation show calpain inhibition preventing AIF translocation and subsequent neuronal death . AIF release from the mitochondria and translocation into the nucleus causes chromatin condensation, DNA fragmentation and cell death (Susin et al., 1999). An alternative excitotoxic mechanism proposes that the activation of PARP-1, a nuclear DNA repair enzyme, causes the release of AIF in excitotoxicity. PARP-1 knockout mice show reduced AIF translocation after NMDA treatment. Moreover, the PAR polymer generated by overactivation of PARP-1 is required to release AIF (Yu et al., 2006). Many studies implicate a very intricate link between the calpain/AIF and PARP-1/ AIF .Cells treated with a DNA alkylating agents or Ca2+, mediated NO production which ultimately produces peroxynitrite a highly oxidant molecule capable of directly damaging DNA activated PARP-1. This leads to release of PAR polymers into the cytoplasm. The PAR polymers activate calpains by some unknown mechanism, this leads to calpain activation. Calpains cause the release of an inactive form of AIF and subsequent cell death. However AIF may not be required at all PAR-mediated cell deaths (Wang et al., 2009).

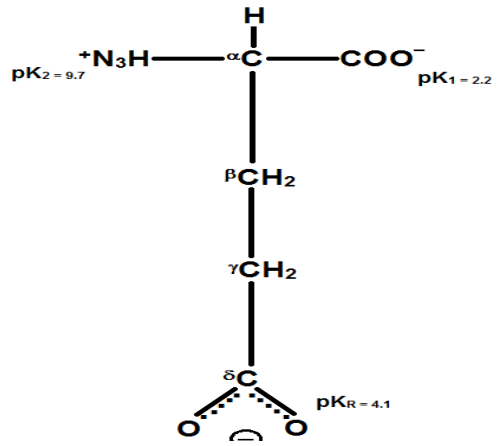
Therapeutic Directions

The NMDAR provides number of sites that can be exploited pharmacologically, including the ion channel pore, the glutamate-binding site, the glycine-binding site and the polyamine interaction site. Channel pore blockers such as MK-801 (dizolcipine), Memantine, Cerestat, Dextromethorphan and its metabolite Dextrorphan reduce Ca2+ entry. However they have many drawbacks such as MK-801 induces PCP-like effects, including cataplexy, locomotory disturbances eventually resulted in its discontinuation. AMPA receptor antagonists appeared more effective in comparison to NMDAR antagonists. AMPAR antagonists such as NBQX, YM872 initially demonstrated neuroprotective effects in various animal models. However, tolerability trials in humans showed side effects such as sedation and euphoria. Taken together, the clinical experience with glutamate receptor manipulation has been poor, toxicity being a large obstacle. Other approaches to neuroprotection moved intracellularly to the generation of free radicals. There are three major classes of free radical scavengers under development: free radical spin traps (nitrone-based), the 21-aminosteroids (also known as lazaroids) and glutathione peroxidase mimics. Unfortunately, like the compounds described above, despite promising animal studies, these drugs have not yet been proven efficacious in humans. Many drugs have been developed to interrupt the glutamate excitotoxic pathway towards neuronal injury (Adam-Vizi, 2000; Gagliardi, 2000). One strategy is the upstream attempt to decrease glutamate release. Lamotrigine, a Na+ channel blocker inhibits presynaptic voltage-dependent Na+ channels, reducing overall excitability and release of neurotransmitter glutamate, GABA, and acetylcholine. However, it failed to show neuroprotection in various animal brain models. BW619C89, a derivative of lamotrigine, showed various neuropsychiatric effects such as reduced consciousness, confusion, hallucinations etc. Similarly, calpain inhibitors against glutamate induced neurotoxicity have also failed to show any protective effects. NCX inhibitors have shown both neuroprotection and worsening of the area after stroke (Pignataro et al., 2004). However, possible neuroprotective and neurodegenerative effects of NCX remain to be determined.

Conclusion

Our understanding of glutamate excitotoxicity has come a long way since its proposal in the 1950s. Decades of research has outlined not only excitotoxic mechanisms but also the manipulation of these pathways in animal systems. Glutamate excitotoxicity is the final common pathway resulting in acute neuronal death for many seemingly unrelated disorders, including ischemia, hypoxia, traumas, Parkinson's syndrome, Alzheimer's dementia, Huntington's disease as well as in chronic neurodegenerative or neurometabolic diseases. Information generated from past few years of intense research work in this field has clearly influenced our basic understanding of the process, but we have not yet fully understood all the complexities involved in excitotoxicity as evidenced by our failure to provide neuroprotection in the clinical setting. Despite intense research into the mechanisms of excitotoxicity, the actual intracellular mechanisms responsible for neuronal death are still being elucidated. One major obstacle is heterogeneity of neurodegeneration following glutamate application. Given that cell invasion constitutes a complex and highly coordinated biology, the disruption of only one pathway may not be sufficient to derail the process. Despite these setbacks, there are exciting new avenues of research targeting this cascade. We hope that glutamate excitotoxicity cascade will lead to novel therapies for neurological diseases. Development of

glutamate antagonists for blocking disease-specific subtypes of glutamate receptors is thus a major area of paramount importance.



Typical skeleton structure of glutamate; consisting of a central alpha carbon (Cα) bonded to a carbonyl group (COOH) on one side and an amino group (H3N) on the other side, characterized by a distinctive molecular group CH2CH2COOH attached to the α-carbon carboxyl group, side chain carboxyl group designated as δ-carboxyl group , becomes fully ionized at neutral pH as COO-

Figure I: Structure of Glutamate

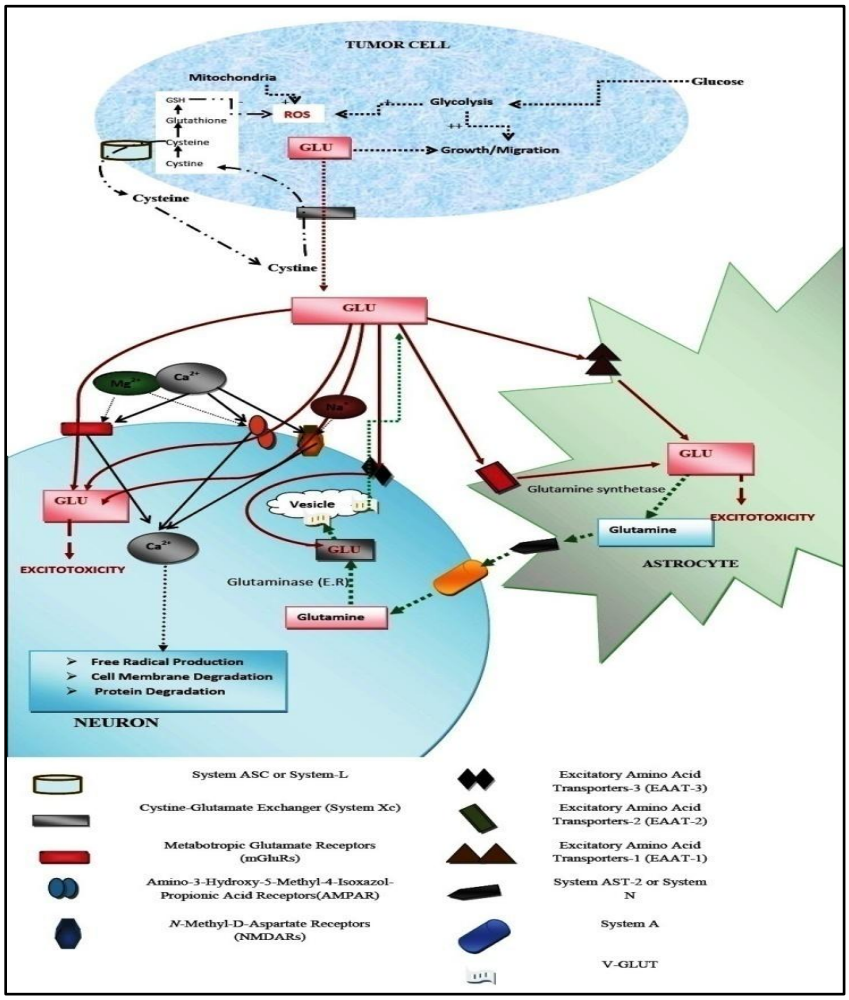


Figure II: Cellular mechanism of glutamate induced excitotoxicity in neurological disorders(© Henah balkhi**).**

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